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(54) Title: ADIPONECTIN FOR TREATING CARDIAC DISEASE

(57) Abstract: The present invention relates to the use of adiponectin for the manufacture of a medicament for the treatment of acute and chronic heart failure or for the treatment of cardiac dysfunction occurring as a consequence of acute or chronic ischemia. The adiponectin may be used in combination with a second agent capable of down-regulating TNF-alpha production, such as pentoxifylline.

ADIPONECTIN FOR TREATING CARDIAC DISEASE

Field of the Invention.

The present invention relates to methods for treating cardiac diseases involving impairment of cardiac muscle function including acute and chronic heart failure and cardiac muscle dysfunction associated with myocardial ischemia.

Background to the Invention.

Cardiovascular disease represents the major cause of death in western societies. The genesis of cardiovascular disease often lies in the development of atheromatous plaque in the coronary vasculature. Over time the developing plaque leads to myocardial ischemia, which can be either chronic (stable angina) or acute (unstable angina and myocardial infarction). For all patients with ischemic heart diseases including those who have survived myocardial infarction, there is a very significant risk of developing heart failure where the heart undergoes remodelling (through hypertrophic growth) and the cardiac muscle becomes dysfunctional. Significant improvements have been made in the last 50 years in the pharmacological therapy of cardiovascular diseases, yet ischemic heart disease and heart failure still accounts for a large burden of patient mortality, morbidity and economic burden. Thus, there is a significant unmet medical need for novel drugs that can improve the function of cardiac muscle under conditions of acute ischemia, cardiac hypertrophy and heart failure.

While under non-stress conditions the heart uses free fatty acids as its primary energy source, under stress conditions such as ischemia, hypertrophy or failure, the heart switches to glucose as its primary metabolic fuel (Opie, 1996; 1999; Lehman and Kelly, 2002).

Under acute ischemia the utilisation of glucose allows the heart to generate ATP more efficiently and it has been shown that interventions which further enhance glucose metabolism (such as infusion of glucose insulin potassium (GIK) solution) confer a clinical benefit in patients suffering acute myocardial infarction (Opie, 1999). Thus, a therapy which acts directly on myocytes to enhance insulin sensitivity would be expected to provide further benefit.

While the switch from fatty acids to glucose as a primary fuel may confer benefits to the acutely ischemic heart, there is growing evidence that this may not be beneficial long term in the failing/remodelling heart. Thus, there is evidence that in patients with hypertensive ventricular hypertrophy and idiopathic cardiomyopathy, there is a sustained reduction in fatty acid oxidation and increased glucose utilisation (Lehman and Kelly, 2002; de las Fuentes *et al.*, 2003; Davila-Roman *et al.*, 2002). Indeed, reduced myocardial fatty acid metabolism has been shown to be an independent predictor of left ventricular mass in hypertension and left ventricular dysfunction (de las Fuentes *et al.*, 2003). This long term suppression of fatty acid oxidation which occurs in the remodelling and failing heart is likely to be detrimental through myocardial energy starvation and the build up of toxic intracellular lipids. Thus, a therapy which is able to return the heart to a more energy efficient state and reduce the progression of maladaptive hypertrophy would be desirable. Furthermore, myocytes from ischemic and failing myocardium elaborate pro-inflammatory and pro-hypertrophic cytokines such as TNFalpha and IL-6. These mediators inhibit contractility, encourage maladaptive hypertrophic growth and stimulate myocyte loss through apoptosis. Thus, there is a need for novel agents which act directly on myocytes to inhibit the elaboration of these local mediators thereby improving the function of acutely ischemic

myocardium and slowing the progression of heart failure in individuals suffering from or at risk of developing the latter condition.

Adiponectin is a protein of about 30 kDa which is classified as an adipokine. Adipokines are produced by fat-cells and include TNFalpha, leptin, resistin, and plasminogen activator inhibitor-1.

EP-A-1033134 (based on WO99/21577) describes the level of adiponectin (also termed apM1) in the plasma of 24 male and 10 female patients with coronary artery disease. It was reported that the levels were low and the authors concluded that adiponectin is a marker of the onset and progression of arteriosclerosis. EP-A-1033134 reports that adiponectin is produced exclusively in adipose tissue, and that it has smooth muscle growth inhibitory activity. The reference also reports that adiponectin inhibits the production of the adhesion molecules VCAM-1, ELAM-1 and ICAM-1 in cultures of human aortic vascular endothelial cells. The reference proposes that compositions of adiponectin might be used in the treatment of arteriosclerosis.

Disclosure of the Invention.

The prior art reports regarding the action of adiponectin, and the proposals of the art regarding its utility, are based on the understanding that adiponectin is expressed exclusively in fat tissue and can have beneficial effects on smooth muscle and endothelial cells. However, in cardiac dysfunction associated with acute ischemia, ventricular hypertrophy and/or heart failure, the principal cell type which is dysfunctional is the cardiac muscle cell (myocyte), which is responsible for the contractile function of the myocardium. Abnormal myocyte contractility, abnormal myocyte growth, myocyte elaboration of

inflammatory mediators, abnormal myocyte metabolism and myocyte death (through apoptosis or necrosis) are all linked to cardiac dysfunction in heart failure and under conditions of acute cardiac ischemia. The present invention stems from discoveries in this field.

We have investigated the expression of adiponectin in a range of human tissue types and have found that adiponectin is in fact highly expressed in cells of the human heart, in particular in atrial and ventricular myocytes and in coronary vascular endothelium. Immunohistochemistry backed up with Western blotting confirms the presence of adiponectin protein in human heart tissue. The immunohistochemistry demonstrated specific myocyte and endothelial localisation. The data provide evidence of significant direct local production of adiponectin in heart tissue.

We have further demonstrated that adiponectin down-regulates the production of TNF-alpha from myocardium with the greatest inhibition at the highest dose of adiponectin (25 μg/ml) tested, reducing the levels of TNF-alpha by 39%. These effects of adiponectin occur via stimulation of specific adiponectin receptors which we have also shown are expressed in the left ventricle of the human heart.

Our findings indicate that adiponectin plays a significant role in cardiac muscle function and therefore may be useful in the treatment of a much broader range of cardiac disease than previously proposed. Local production of adiponectin is likely to lead to beneficial direct local effects on cardiac muscle (myocyte) function especially in ischemic, hypertrophied and/or failing myocardium. These beneficial effects stem from the ability of adiponectin to improve insulin sensitivity, improve myocardial (myocyte) glucose utilisation, enhance myocardial

(myocyte) fatty acid oxidation. AMPK (AMP-activated protein kinase) activation is thought to be involved in mediating these effects. Importantly AMPK expression and activation is reduced in the failing heart.

Based on the aforementioned biology of adiponectin and that the failing heart exhibits characteristic changes in its metabolic function, the present findings are consistent with the notion that local production of adiponectin is likely to lead to beneficial direct effects on cardiac muscle function, especially in heart failure. These beneficial effects stem from the ability of adiponectin to primarily act via AMPK to improve insulin sensitivity, improve myocardial glucose utilisation and enhance fatty acid oxidation. Additionally adiponectin inhibits the production of pro-inflammatory/negative inotropic cytokines, specifically TNF-alpha from the myocardium probably by inhibiting the activation of the NFkappaB pathway, and may also have beneficial effects on pathways leading to remodelling of the heart, such as hypertrophy and/or apoptosis.

Thus agents, such as adiponectin that can modulate and increase glucose utilisation/insulin sensitivity and reduce cardiac/circulating levels of free fatty acids maybe useful for the treatment of heart failure.

In addition, the direct local production of adiponectin by myocytes indicates that adiponectin would be suitable for cardiac gene therapy applications.

In a first aspect, the invention provides a method of treatment of acute and chronic heart failure which method comprises administering an effective amount of adiponectin to a patient in need of treatment.

In another aspect, the invention provides a method of the treatment of cardiac dysfunction occurring as a consequence of acute or chronic ischemia which method comprises administering an effective amount of adiponectin to a patient in need of treatment.

In another aspect, the invention provides a method of treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia, which method comprises administering heart muscle tissue of a patient in need of treatment an effective amount of a gene therapy vector capable of expressing adiponectin in a myocyte.

The invention further provides the use of adiponectin or a gene therapy vector capable of expressing adiponectin in a myocyte for the manufacture of a medicament for treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia. Reference herein to the various embodiments of the methods of the invention also include the use of adiponectin for the manufacture of a medicament for use in such methods.

In another aspect, the invention provides a method of regulating TNF-alpha production in a heart muscle cell which comprises bringing adiponectin or an agonist of adiponectin into contact with said cell. The method may be performed *in vitro* or *in vivo*.

In a further aspect, the invention provides the use of adiponectin in combination with second agent capable of down-regulating TNF-alpha production for the manufacture of a medicament as a combined preparation for simultaneous, separate or sequential use in the treatment of acute and chronic heart

failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

Further, the invention provides a method of treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia, which method comprises administering to a patient in need of treatment an effective amount of adiponectin in combination with second agent capable of down-regulating TNF-alpha production.

The invention also provides a composition comprising adiponectin in combination with second agent capable of down-regulating TNF-alpha production.

In a further aspect, there is provided a method of down-regulating the production of TNF-alpha in the myocardium, which method comprises administering to a subject an effective amount of adiponectin. Such a method may be practiced in a subject who has acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

In such methods, a second agent capable of down-regulating TNF-alpha production may be administered simultaneously, separately or sequentially with the adiponectin.

The second agent in the above-mentioned aspects of the invention may be pentoxifylline.

Description of the Drawings

Figure 1 shows adiponectin mRNA expression profile in control, IDC and ICM human left ventricular myocardium.

Figure 2 shows the effect of adiponectin on TNF-alpha release from rat myocardium.

Figure 3 shows the expression (copy number) of mRNA for the three known adiponectin receptors in human left ventricular myocardium.

Detailed Description of the Invention

Acute and chronic heart failure.

By acute and chronic heart failure, it is meant that the patients are suffering from impaired contractile ability of the heart such that circulatory demands cannot be met. Clinically, this is likely to be diagnosed by breathlessness, fatigue, oedema and impaired cardiac function (reduced ejection fraction) following echocardiography. Such patients are likely to show (via echocardiography) marked cardiac remodelling associated with either concentric or eccentric enlargement of the left ventricle. Such patients are likely to show a background of one or more of ischemic heart disease, myocardial infarction, hypertension, vascular disease, myocarditis, or genetic mutation in key cardiac structural or contractile genes. Thus the methods of the invention may be used to treat a subject with any one of these conditions or symptoms associated with acute or chronic heart failure. These conditions often lead either rapidly or more commonly progressively to cardiac structural remodelling, diastolic dysfunction, systolic dysfunction and the clinical conditions or symptoms described above.

Cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

By cardiac dysfunction occurring as a consequence of acute or chronic ischemia, it is meant a sudden loss in cardiac contractile function associated with an episode of acute ischemia. For example, in patients suffering acute myocardial infarction, in the immediate 1-2 week period following the infarction, the function of the remaining viable cardiac muscle is impaired as result of both irreversible loss of myocytes and

reversible damage to cardiac myocytes. Such reversible injury is often referred to as "stunning". This stunning type reversible injury, resulting in acute cardiac dysfunction, can also be seen in patients undergoing angioplasty or thrombolysis (to clear blocked coronary arteries), in patients with unstable angina, in patients undergoing stress-induced ischemia or in patients undergoing coronary artery bypass grafting.

Thus the method of the invention may be practiced on a patient undergoing angioplasty or thrombolysis or in a patient undergoing coronary artery bypass grafting. The treatment may include administering adiponectin or a vector encoding adiponectin at the time of surgery, or post-operatively, e.g. within 7 days, preferably within 24 hours of these procedures.

In particular, the invention may be performed on a subject who has suffered a myocardial infarction. Preferably the invention is performed on a patient who has had a myocardial infarction within the previous 14 days, preferably within the previous 7 days, and most preferably within the previous 48 hours. The patient may be one who has no previous history of myocardial infarction, i.e. the treatment is in respect of a first myocardial infarction, and preferably the treatment has commenced within the above-mentioned time period.

The methods of the invention may be performed on a subject who has not previously been treated with adiponectin, e.g. for the symptoms of arteriosclerosis or atherosclerosis, or diabetes.

Adiponectin

Adiponectin is a 30 kDa protein, the human form of which is available as Genbank entry NP_004788, encoded by a DNA sequence available as Genbank entry NM_004797. Although the use of full length mammalian, preferably human, adiponectin is envisaged in the present invention, it will also be understood that the term

includes fragments of adiponectin which retain the ability to exert beneficial activity on human myocardium *in vitro*. Adiponectin, including fragments thereof, may be conjugated to additional peptide sequences designed to target the protein to specific cell types or to aid protein transfection into cells, such as the TAT sequence or the translocation sequence derived from the Drosophila melanogaster antennapedia protein.

Fragments of adiponectin include globular domain fragments comprising residues 101-242 of the human adiponectin sequence, or the corresponding residues of mammalian homologues. Examples of such fragments include adiponectin 52-244, 58-244, 82-244 and 101-244.

Adiponectin and its fragments may be conjugated to polymers, such as polyethylene glycol (PEG) designed to extend the half-life of the protein in the environment of a human body.

Adiponectin and its fragments may be produced by chemical synthesis or recombinant DNA methods, as described in EP 1033134, the contents of which are incorporated herein by reference. For example, a nucleic acid sequence encoding adiponectin may be operably linked to a promoter to provide for expression of adiponectin in a host cell compatible with the promoter. Such a host cell may be a eukaryotic or prokaryotic host cell.

Suitable prokaryotic host cells include *E. coli* and *B. subtilis*, and vectors suitable for use in these or other prokaryotic cells are well known in the art. Preferably however a eukaryotic cell may be used, such as a yeast cell or a mammalian cell. Of the latter, Chinese hamster ovary (CHO) cells are widely used for the production of many different proteins and may be used here.

The host cell will be cultured under conditions to provide for production of adiponectin, and the adiponectin recovered from the host cell and purified for pharmaceutical use.

In addition, adiponectin agonists capable of binding an adiponectin receptor present on heart muscle tissue may be used. Reference herein to adiponectin thus includes such agonists.

Pharmaceutical compositions containing adiponectin may be formulated for any suitable route and means of administration. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For solid compositions, conventional non-toxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. Adiponectin may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc, adiponectin

and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

Dosage forms or compositions containing adiponectin in the range of 0.0001 to 70 weight % with the balance made up from non-toxic carrier may be prepared.

The amount of adiponectin to be administered will be dependent upon the nature of the patient and his condition, though by way of general guidance the dosage will be selected so that the resulting blood concentration of the adiponectin will be about 1 to 200 μg per ml.

The pharmaceutical composition may additionally comprise a second agent capable of down-regulating TNF-alpha production together with a pharmaceutically acceptable carrier.

Gene Therapy Vectors.

As an alternative to administration of adiponectin, a gene therapy vector may be administered. US patent 6,228,844 - the contents of which are incorporated herein by reference - teaches that naked DNA can be taken up by vertebrate cells, particularly muscle cells. Thus a polynucleotide which has all the genetic information necessary for expression by a target cell, such as promoters and the like, and a coding sequence for adiponectin, may be administered. These polynucleotides can be administered to the vertebrate by any method that delivers injectable materials to cells of the vertebrate, most preferably directly to the myocytes. However, it will be understood that reference herein to "administering to the heart muscle tissue" will include methods where the vector is administered to another part of the body but in a form where at least some of the vector so administered is transported to the heart muscle tissue, taken up and expressed.

Thus for example the polynucleotides may be delivered by direct injection to the myocytes or via a catheter, for example a cardiac catheter for delivery of substances to the arteries of the heart.

A naked polynucleotide is injected or otherwise delivered to the animal with a pharmaceutically acceptable liquid carrier. In preferred applications, the liquid carrier is aqueous or partly aqueous, comprising sterile, pyrogen-free water. The pH of the preparation is suitably adjusted and buffered. The DNA may optionally be associated with a liposome.

Alternatively, various viral vectors which can be utilized for gene therapy may be used to deliver the DNA or nucleic acid in the form of RNA. Such vectors include adenovirus, herpes virus, lentivirus, vaccinia or a retrovirus. Examples of retroviral

vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). When the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) can be utilized. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

The DNA sequences used may be sequences which do not integrate into the genome of the host cell. These may be non-replicating DNA sequences, or specific replicating sequences genetically engineered to lack the genome-integration ability.

The polynucleotide material delivered to the cells in vivo can take any number of forms, and the present invention is not limited to any particular form.

Where the polynucleotide is to be DNA, promoters suitable for use in various vertebrate systems are well known. For example, for use in murine systems, suitable strong promoters include RSV LTR, MPSV LTR, SV40 IEP, and metallothionein promoter. In humans, on the other hand, promoters such as CMV IEP may advantageously be used. More preferably, promoters driving myocyte specific over-expression of adiponectin could be used. Such promoters include alphaMHC and MLC2v promoters.

It may be desirable to provide for transitory polynucleotide synthesis in the cells, so that adiponectin is expressed for a few hours or days to facilitate treatment of the cell, which then reverts back to normal levels of adiponectin production.

Polynucleotides may be prepared in unit dosage form in ampoules, or in multi-dose containers. The polynucleotides may be present

in such forms as suspensions, solutions, or emulsions in oily or preferably aqueous vehicles. The compositions per unit dosage, whether liquid or solid, may contain from 0.1% to 99% of polynucleotide material. The dosage to be administered depends to a large extent on the condition and size of the subject being treated as well as the frequency of treatment and the route of administration. The concentration of polynucleotide in the formulation is from about 0.1 μg/ml to about 20 mg/ml.

Gene therapy vectors may be administered to a patient receiving treatment with a second agent capable of down-regulating TNF-alpha production. Thus the invention provides the use of an agent, other than adiponectin, which is capable of down-regulating TNF-alpha production, for the manufacture of a medicament for the treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia in a subject, wherein said subject has received, or is receiving, or is about to receive, a gene therapy vector in accordance with the present invention.

The invention further provides a method of treatment of a subject who has acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia, which method comprises administering to the subject a gene therapy vector in accordance with the present invention together with a second agent capable of down-regulating TNF-alpha. The two products (vector and second agent) may be administered separately or sequentially, as defined herein.

Summary

The data in the accompanying examples show that adiponectin reduces the production of the inflammatory cytokine TNF-alpha in the human myocardium. This finding is especially significant in view of the fact that TNF-alpha levels are elevated in the

plasma of patients with severe chronic heart failure and the level of TNF-alpha correlates with functional class (Levine *et al*, 1990; Torre-Amione *et al*, 1996). Additionally, TNF-alpha has negative inotropic properties. Recent small clinical studies in idiopathic dilated cardiomyopathy (Skudicky *et al*, 2001) and ischemic cardiomyopathy (Sliwa *et al*, 2004) have demonstrated that the use of pentoxifylline, an inhibitor of TNF-alpha production, is capable of having additive beneficial effects above the current treatments for heart failure, including ACE inhibitors and β-blockers. Over a six month period, pentoxifylline caused a significant improvement in functional class, ejection fraction and markers of apoptosis (Fas/Apo-1) in patients from both disease groups. These studies demonstrate that immunomodulating agents, particularly ones that inhibit TNF-alpha production are beneficial at a clinical level. Thus, as it has now been found that adiponectin down-regulates TNF-alpha production in myocardial tissue, adiponectin may be used to improve cardiac function in the context of heart failure by reducing local TNF-alpha levels. The adiponectin may be used in conjunction with other TNF-alpha-modulating agents, such as pentoxifylline.

Accordingly, the present findings indicate that the use of adiponectin will allow second agent capable of down-regulating TNF-alpha production to be used more effectively, where adiponectin is administered with second agent capable of down-regulating TNF-alpha production.

In a further aspect, the method provides adiponectin and second agent capable of down-regulating TNF-alpha production, as a combined preparation for simultaneous, separate or sequential use in therapy for the treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

The invention further provides the use of adiponectin for treating acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia in a subject, wherein the subject has received treatment with a second agent capable of down-regulating TNF-alpha production at the time of adiponectin administration. Alternatively, the invention provides the use of said second agent for treating acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia in a subject, wherein the subject has received treatment with adiponectin.

By "simultaneous " administration, it is meant that adiponectin and the second agent capable of down-regulating TNF-alpha production are administered to a subject in a single dose by the same route of administration.

By "separate" administration, it is meant that adiponectin and the second agent capable of down-regulating TNF-alpha production are administered to a subject by two different routes of administration which occur at the same time. This may occur for example where one agent is administered by infusion and the other is given orally during the course of the infusion.

By "sequential" it is meant that the two agents are administered at different points in time, provided that the activity of the first administered agent is present and ongoing in the subject at the time the second agent is administered. Generally, a sequential dose will occur such that the second of the two agents is administered within 48 hours, preferably within 24 hours, such as within 12, 6, 4, 2 or 1 hour(s) of the first agent.

The agents will be formulated appropriately for their desired route of administration. The agent or pharmaceutical composition comprising the agent may be administered to a subject by any convenient route of administration, e.g. systemically or directly to the heart.

Preferably the second agent is pentoxifylline. Suitable doses of pentoxifylline can be in the range of from 0.1 to 1200 mg/kg/day. The precise dose may vary depending on the route of administration. For i.v. administration, a dose range of from 0.1 to 120 mg/kg/day is preferred. For oral administration, a dose range of from 1 to 1200 mg/kg/day is preferred.

The invention is illustrated by the following examples:

Example 1a: Expression of adiponectin mRNA in normal tissue. The expression of adiponectin mRNA was determined in 72 human tissues using quantitative reverse transcription polymerase chain reaction (QRT-PCR). Total RNA was isolated from human left ventricular myocardium. RNA isolation was achieved using TriZol™, a commercially available solution of phenol and guanidine isothiocyanate, according to the protocol described by the manufacturer (Life Technologies). Samples of RNA were used in this study only if intact 18S and 28S ribosomal RNA were detected by gel electrophoresis and if genomic DNA formed less than 10% of the total nucleic acid sample. Total RNA samples were annealed to the primer probe sequence plus a glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession no. P04406) primer and reverse transcribed using MuLV reverse transcriptase. Quantitative sequence detection was carried out on the resulting cDNA.

The applicants have developed protocols for quantitative analysis of mRNA expression using the ABI prism 7700 Sequence

Detection System (Perkin Elmer). Details of the system are set out in WO00/05409. In brief, the system uses fluorogenic probes to generate sequence specific fluorescent signals during PCR. The probes are oligonucleotides with fluorescent reporter and quencher dyes attached. While a probe is intact, the intensity of reporter fluorescence is suppressed by a quencher. When a probe forms part of a replication complex during the PCR process, the quencher is separated from the reporter dye resulting in a increase in fluorescence which is then detected by the ABI 7700 sequence detector. The ABI 7700 has a built in thermal cycler, and a laser directed at each of the 96 sample wells via bi-directional fibre optic cables. Emitted fluorescence through the cables to a detector where emissions which fall between 520nm and 660nm are collected every few seconds. The system software analyses the contribution of each component dye to the experiment spectrum, and normalises the signal to an internal reference dye. The peaks of these normalised 'reporter' values (Rn) are then plotted against thermal cycle number to produce an amplification plot - to allow visualisation of the extent of PCR product generation.

The starting copy number of a target sequence (Cn) is established by determining the fractional PCR cycle number (Ct) at which a PCR product is first detected - the point at which the fluorescence signal exceeds a threshold baseline. Therefore the lower a Ct value the greater the Cn. Quantification of the amount of target mRNA in each sample is established through comparison of the experimental Ct values with standard curves for the target sequence which are constructed during each experiment.

Primer probe sets were specifically designed for the detection of adiponectin mRNA. Off-line homology searches revealed no significant matches with gene sequences logged at Genbank.

Forward and reverse primer and probe sequences for adiponectin were as follows:

Forward	CCACTATGATGGCTCCACTGGTA	(SEQ ID NO:1)
Reverse	TAGACTGTGATGTGGTAGGCAAAGTAG	(SEQ ID NO:2)
Probe	ATTCCACTGCAACATTCCTGGGCTGT	(SEQ ID NO:3)

GAPDH primer probe sets were as follows

Forward	GAAGGTGAAGGTCGGAGTCAAC	(SEQ ID NO:4)
Reverse	CAGAGTTAAAAGCAGCCCTGGT	(SEQ ID NO:5)
Probe	TTTGGTCGTATTGGGCGCCT	(SEQ ID NO:6)

Reaction conditions were optimised using genomic DNA as a template and a primer probe concentration grid followed by a probe concentration gradient experiment. Primer concentrations were selected to give the most efficient amplification of gene product; i.e. those which generate a low threshold cycle and a relatively high accumulation of fluorescence. These optimal primer concentrations were then used to select the optimum probe concentration.

The results confirmed that adiponectin is highly expressed in adipose tissue (mean mRNA copy number = 73,334) but also revealed the novel observations that adiponectin is highly expressed in cardiac atria (mean mRNA copy number = 24,268), ventricle (mean mRNA copy number = 1,671) and coronary artery (mean mRNA copy number = 16,929). Significant expression was also observed in skeletal muscle (mean mRNA copy number = 1186), pulmonary artery (mean mRNA copy number = 607) and renal artery (mean mRNA copy number = 637) but <u>not</u> in cerebral artery (mean mRNA copy number = 24).

The expression of adiponectin appears to be specific to certain tissue as only low levels or no expression was observed in a wide range of other tissue types, including adrenal-gland, cerebral and choroid-plexus blood-vessels, various brain tissue, caecum, blood-mononuclear cells, colon, duodenum, fallopian-tube, gallbladder, ileum, jejunum, various kidney tissue, liver parenchyma, lung parenchyma, tonsil, oesophagus, ovary, pancreas, pineal-gland, pituitary-gland, placenta, prostate, rectum, foreskin, spinal-cord, spleen, various stomach tissue, testis, umbilical-cord, ureter, cervix, myometrium and vas-deferens.

Thus adiponectin is expressed in cardiac muscle tissue, which has not previously been appreciated in the art.

Example 1b: Adiponectin mRNA expression profile in control, IDC and ICM human left ventricular myocardium.

Samples of human left ventricular free wall were selected based on the donor's clinical history and histological examination of the ventricular samples by a qualified pathologist. The tissues were classified as Group 1, control, non diseased (n=13); Group 2, idiopathic dilated cardiomyopathy (IDC) (n=13); Group 3, ischemic cardiomyopathy (ICM) (n=12). Quantitation of adiponectin mRNA was performed as described above in Example 1a. Adiponectin mRNA copy number is shown in Figure 1 ("Cont" = Control (non-diseased); IDC = idiopathic dilated cardiomyopathy; ICM = ischemic cardiomyopathy). Each bar represents the geometric mean mRNA copy number with 95 % confidence interval on a log scale.

Example 2: Protein expression profiling.

The expression of adiponectin was studied using a specific monoclonal antibody (MAB3604 from Chemicon International) in cardiac tissue derived from non-diseased donors, donors with end

stage idiopathic cardiomyopathy and donors with ischemic heart failure.

Formalin-fixed, paraffin-embedded transverse and oblique sections (5-7μm thick) were cut from n=4 donors of heart left ventricle from each of the three study groups (i.e. n=12 donors in total) plus n=1 breast as a positive control tissue for adiponectin. Sections were then de-paraffinised in xylene and re-hydrated through a series of decreasing concentrations of alcohol to PBS. Subsequently sections underwent heat-mediated antigen retrieval in boiling vector antigen unmasking solution for 3 x 5 minutes in a microwave oven and were then washed in PBS. Following this, sections were immersed in 0.3% hydrogen peroxide / 100% methanol for 20 minutes at room temperature to quench endogenous peroxidases, washed in PBS and then incubated with 1% normal horse serum / PBS for 15 minutes at room temperature, to block non-specific protein binding sites. The adiponectin primary antibody was made up in 1% normal horse serum / PBS and applied to sections at a concentration of 2 μg/ml for 1 hour at room temperature in a humidified chamber. An adjacent section from each sample was processed in parallel as a non-immune negative control, in this instance, mouse IgG1κ at 2 μg/ml. Following further washes in PBS, Vectastain Universal Elite ABC kit secondary and tertiary antibodies were made up according to manufacturer instructions and sequentially applied to sections for 30 minutes each at room temperature, with PBS washes in between. Signal was detected using Vector DAB peroxidase substrate, which was applied to sections for 2.5 minutes at room temperature after which sections were rinsed in distilled water. Sections were then counterstained in Mayers Haematoxylin for 1 minute, rinsed in tap water, dehydrated through increasing concentrations of alcohols, cleared in xylene and coverslipped using DPX glue. Images were captured using an Olympus BX51 microscope and Olympus DP12 digital camera.

Expression of adiponectin was found within the cytoplasm of myocytes in each of the study groups, i.e. non-diseased, end stage idiopathic cardiomyopathy and ischemic heart failure patients. The staining was often concentrated around the plasma membrane. However, the occasional myocyte was relatively negative. Staining seen between muscle fibres cut in a more transverse rather than oblique plane, appeared to be immuno-reactivity of small capillaries. Indeed, vessel endothelium in larger blood vessels was also seen to express adiponectin. Adipocytes within adipose tissue attached to certain cardiac tissue samples were also immunoreactive, as expected. Staining intensity varied between donors within the same study group. No staining was found using the non-immune control.

Example 3: Protein expression profiling by western blotting. Total protein was isolated from human left ventricular free wall using a protein extraction kit (Calbiochem) and quantified using a non-interfering protein assay (Calbiochem). The protein (40 μg) was mixed 1:1 with Laemmli 2x solution and denatured by heating at 95°C for 10 minutes. The samples were run down a 10% gel (Biorad) according to the manufactures instructions with appropriate molecular weight markers. The protein was then transferred onto a PVDF membrane and PONCEAU-S stained to insure that the gel and transfer had worked properly. The blots were blocked for 1 hour with 2 % MARVEL, 1 % BSA. After washing they were exposed to the primary antibody, MAB3604, (1:2500 dilution) for 1 hour. Binding of the primary antibody was then detected using ECF (Amersham) according to the manufacturers protocol and detected using a STORM fluorimager. Immunoreactivity was observed at 28-30 KDa which corresponds to adiponectin in protein extracted from human heart left ventricle (n=2).

Example 4: Effect of adiponectin on TNF-alpha release from myocardium.

Whole rat hearts were cut into segments of ~1-2mm^2 in cold PBS w/o calcium (on wet ice). The heart segments were placed in fresh PBS w/o calcium and left for 20min at room temperature and then transferred to 24 well plates (~150 mgs/well). The samples (n=5) were pre-treated for 6 hours with media, vehicle; 0.1% BSA, or 1, 10, 25 µg/ml adiponectin (R&D Systems). LPS at a final concentration of 1µg/ml or an equal volume of media was added to the wells and incubated for a further 4 hours. The supernatants were removed and stored at -80°C. Figure 2 shows the results obtained. The ordinate scale represents TNF-alpha protein levels with each bar representing the mean + sem.

Example 5: Expression of the adiponectin receptors in human left ventricular myocardium.

Three subtypes of receptor for adiponectin have been identified in the prior art (ADIPOR1 and ADIPOR2 Yamauchi et al., 2003: TNFRSF19 (termed Omoxin in patent WO03/013578). The expression of each of these adiponectin receptors at the mRNA level was determined using the methodology described above in Example 1 except using the following primer probe sets: Figure 3 shows the results obtained. The ordinate scale represents copy number.

Primer probe sets were specifically designed for the detection of ADIPOR1; ADIPOR2; TNFRSF19 mRNA. Off-line homology searches revealed no significant matches with gene sequences logged at Genbank. Forward and reverse primer and probe sequences for each gene were as follows:

Adiponectin receptor 1 (ADIPOR1) has the following synonyms: (Adiponectin receptor protein 1, CGI-45, CGI-45 protein)
Genbank Accession Number: NM_015999
Protein Accession Number: NP_057083

Forward GTTCCTGGGACTTGGCTTGA (SEQ ID NO:7)
Reverse CAAAGCCCTCAGCGATAGTAAAG (SEQ ID NO:8)
Probe TGGCGTCGTGCCCACCATG (SEQ ID NO:9)

Adiponectin receptor 2 (ADIPOR2) has the following synonyms:
(Adiponectin receptor protein 2, FLJ21432, MGC4640)
Genbank Accession Number: NM_024551
Protein Accession Number: NP_078827

Forward GATAGGCTGGTTGATGCTGATG (SEQ ID NO:10)
Reverse GGATCCGGGCAGCATACA (SEQ ID NO:11)
Probe CCAGCCTCTACATCACAGGAGCTGCC (SEQ ID NO:12)

Tumor necrosis factor receptor superfamily, member 19 (TNFRSF19), has the following synonyms:
(Tumor necrosis factor receptor superfamily member 19 precursor (Toxicity and JNK inducer) (TRADE) TAJ, TAJ-alpha, TRADE, TROY, toxicity and JNK inducer)
Genbank Accession Number: Variant 1 NM_018647, variant 2 NM_148957
Protein Accession Number: Variant 1 NP_061117, variant 2 NP_683760

Primer probe set is designed to pick up both variants

Forward AGGGATCGGTCTGGAAACTGT (SEQ ID NO:13)
Reverse GCCACATTCCTTAGACAACTCCAT (SEQ ID NO:14)
Probe TTCCCTGCAACCAGTGTGGGCC (SEQ ID NO:15)

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Claims

1. Use of adiponectin for the manufacture of a medicament for the treatment of acute and chronic heart failure.

2. Use of adiponectin for the manufacture of a medicament for the treatment of cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

3. Use of adiponectin in combination with second agent capable of down-regulating TNF-alpha production for the manufacture of a medicament as a combined preparation for simultaneous, separate or sequential use in the treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

4. Use according to claim 3 wherein said second agent is pentoxifylline.

5. Use of a gene therapy vector capable of expressing adiponectin in a myocyte for the manufacture of a medicament for the treatment of acute and chronic heart failure or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

6. The use of any one of claims 1 or claim 3 to 5 wherein the treatment is in a patient showing symptoms associated with acute or chronic heart failure.

7. The use of any one of claims 1 to 5 wherein the treatment is in a patient who has suffered a myocardial infarction within the previous 14 day period.

8. The use of any one of any one of claims 1 to 5 wherein the treatment is in a patient who has had no previous history of myocardial infarction.

9. The use of any one of the preceding claims wherein said patient has not previously been treated with adiponectin.

10. A method of treatment of acute and chronic heart failure which method comprises administering an effective amount of adiponectin to a patient in need of treatment.

11. A method of the treatment of cardiac dysfunction occurring as a consequence of acute or chronic ischemia which method comprises administering an effective amount of adiponectin to a patient in need of treatment.

12. A method of treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia, which method comprises administering to a patient in need of treatment an effective amount of adiponectin in combination with second agent capable of down-regulating TNF-alpha production.

13. A method according to claim 12 wherein said second agent is pentoxifylline.

14. A method of treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia, which method comprises administering to the heart muscle tissue of a patient in need of treatment an effective amount of a gene therapy vector capable of expressing adiponectin in a myocyte.

15. The method of claim 10, 12, 13 or 14 wherein the treatment is in a patient showing symptoms associated with acute or chronic heart failure.

16. The method of any one of claims 10 to 15 wherein said patient has suffered a myocardial infarction within the previous 14 day period.

17. The method of any one of any one of claims 10 to 15 wherein said patient has had no previous history of myocardial infarction.

18. The method of any one of claims 10 to 17 wherein said patient has not previously been treated with adiponectin.

19. A composition comprising adiponectin in combination with second agent capable of down-regulating TNF-alpha production.

20. The composition of claim 20 wherein said second agent is pentoxifylline.

21. The composition of claim 19 or 20 for use in a method of treatment of the human or animal body.

22. The composition for use according to claim 21 wherein said treatment is the treatment of acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

23. A method of down-regulating the production of TNF-alpha in the myocardium, which method comprises administering to a subject an effective amount of adiponectin.

24. The method of claim 23 wherein the subject has acute and chronic heart failure, or cardiac dysfunction occurring as a consequence of acute or chronic ischemia.

25. The method of claim 23 or 24 wherein a second agent second agent capable of down-regulating TNF-alpha production is administered simultaneously, separately or sequentially with the adiponectin.

26. The composition of claim 20 wherein said second agent is pentoxifylline.

27. A method of regulating TNF-alpha production in a heart muscle cell which comprises bringing adiponectin or an agonist of adiponectin into contact with said cell.

Figure 1

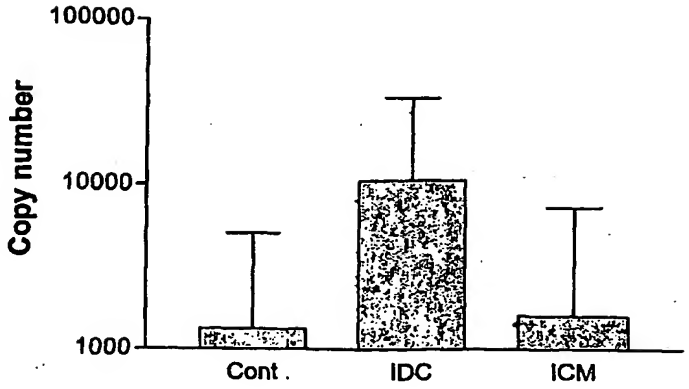
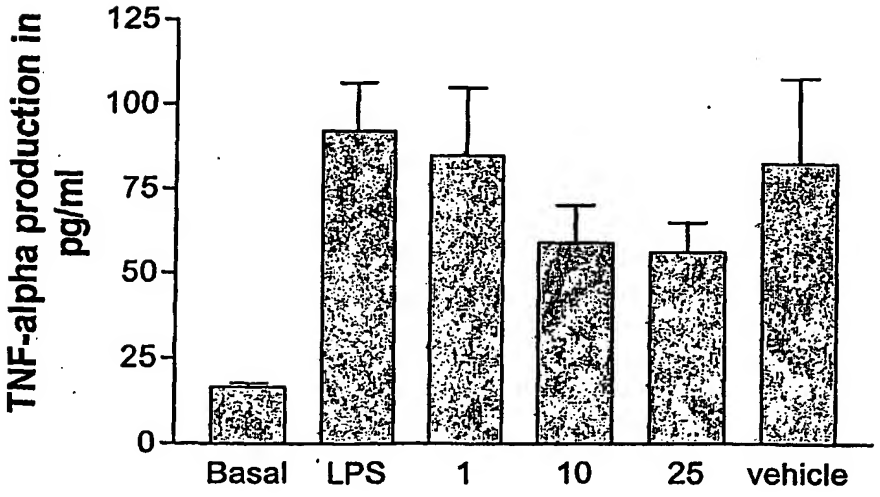


Figure 2



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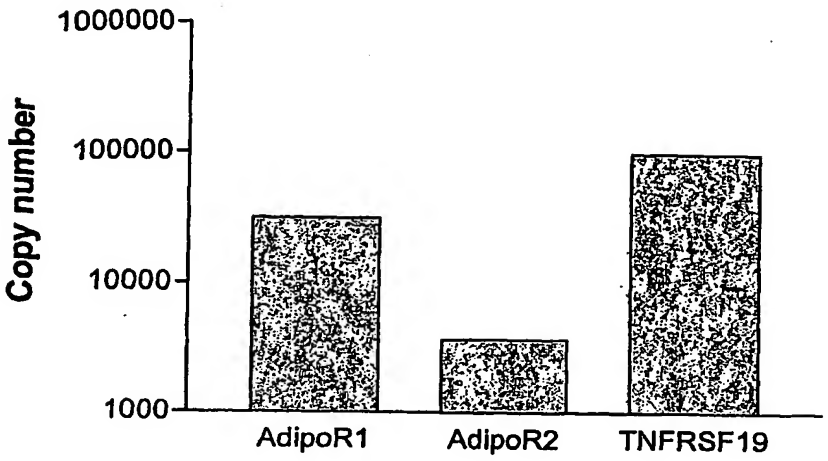
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INTERNATIONAL SEARCH REPORT

Application No
/GB2004/002288

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATSUMOTO KENGO ET AL: "Adiponectin, a novel adipocyte-derived plasma protein, is a protective factor for coronary artery disease." CIRCULATION, vol. 106, no. 19 Supplement, 5 November 2002 (2002-11-05), page II.425, XP009034878 & ABSTRACTS FROM SCIENTIFIC SESSIONS; CHICAGO, IL, USA; NOVEMBER 17-20, 2002 ISSN: 0009-7322	1,2, 5-11, 14-18, 23-25,27
Y	abstract	3,4,12, 13, 19-22,26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2004	09/09/2004

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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/GB2004/002288

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 033 134 A (OTSUKA PHARMA CO LTD) 6 September 2000 (2000-09-06) cited in the application	1,2, 5-11, 14-18, 23-25,27
Y	paragraph '0015! claim 2; example 5	3,4,12, 13, 19-22,26
X	OLAVI UKKOLA ET AL: "Adiponectin: a link between excess adiposity and associated comorbidities?" J MOL MED, vol. 80, 2002, pages 696-702, XP001183047	1,2, 5-11, 14-18, 23-25,27
Y	Conclusions	3,4,12, 13, 19-22,26
P,X	DATABASE WPI Section Ch, Week 200402 Derwent Publications Ltd., London, GB; Class B04, AN 2004-023231 XP002291853 & WO 03/099319 A1 (JAPAN SCI & TECHNOLOGY CORP) 4 December 2003 (2003-12-04) abstract	1,2, 5-11, 14-18, 23-25,27
P,X	DATABASE WPI Section Ch, Week 200359 Derwent Publications Ltd., London, GB; Class B04, AN 2003-627572 XP002291854 & WO 03/063894 A1 (JAPAN SCI & TECHNOLOGY CORP) 7 August 2003 (2003-08-07) abstract	1,2, 5-11, 14-18, 23-25,27
Y	US 5 112 827 A (SAUNDERS J PALMER ET AL) 12 May 1992 (1992-05-12) abstract; claim 11	3,4,12, 13, 19-22,26
Y	DD 264 850 A (ISIS CHEMIE ZWICKAU VEB) 15 February 1989 (1989-02-15) abstract page 1, line 20 - line 22	3,4,12, 13, 19-22,26
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GB2004/002288

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		WO 9921577 A1	06-05-1999
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